Supplementary material and methods and figures:

Report:

Monotherapy efficacy of blood-brain barrier permeable small molecule reactivators of protein phosphatase 2A in glioblastoma

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Supplementary materials and methods

Western blotting and antibodies

Cultured cells were lysed in 2x Laemmli buffer (4% SDS, 20% glycerol, 120mM Tris) and resolved by SDS-PAGE gel (BioRad, Country). Proteins were transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% milk-TBS and incubated with required dilution of primary and 1:5000 dilution of secondary antibody in 5% Milk-TBS-Tween 20 for required duration of time and visualized with odyssey (LI-COR biosciences, Nebraska, USA).

The membrane was blocked using 5% milk in Tris-buffered saline (TBS) and incubated with a primary antibody PME-1 (sc-20086,1:1000), CIP2A (sc-80659,1:500 dilution) and SET (sc-133138,1:1000) from Santa Cruz Biotechnology. Antibodies for Nestin (MAB5326) (1:1000 dilution) was acquired from Merck, SOX2 (3579p) (1:500) From Cell signaling and ARPP-19 (11678-1-AP) (1:250) from Proteintech. Loading control antibodies for β-actin (sc-47778) (1:10,000

dilution) was from Santa-Cruz Biotechnology. Secondary antibodies were purchased from LI-COR, mouse (926-32212) and rabbit (926-68021).

In vitro blood-brain barrier model and permeability measurements

In vitro blood-brain barrier assay was established as previously published (*28*) and described in detail in supplementary materials and methods. Effect of NZ-8-061 and DBK-1154 on *in vitro* BBB permeability was measured by following passive diffusion of small-molecular-weight fluorescent dye sodium-fluorescein (Na-Fl) over time from the blood to the brain side of the inserts. Na-Fl solution (50 µM) in EBM-, 2.5 ml per well, was prepared. The media on the blood side of the inserts was replaced with containing Na-Fl media. 100 µl samples of media were collected from both the blood and the brain side of the inserts at 15 min and transferred to 96-well plate. Results were measured in duplicates and quantified with plate reader with filter set on 480/560 nm. The drugs NZ-8-061 and DBK-1154 were administered in 15 µM concentration 24 hours before application of Na-FI.

Measuring DBK-1154 and NZ-8-061 concentrations with HPLC-MS/MS

100 µl samples of NZ-8-061 and DBK-1154 were collected from both sides of the inserts at 1, 6, 12 and 24 hours. Removed media was replaced with fresh media with drugs after each timepoints. NZ-8-061 and DBK-1154 were administered in 15 µM concentration. Glipizide (Sigma-Aldrich Chemie) was used as an internal standard. Stock solutions (300 µM) and working solutions were prepared in dimethyl sulfoxide (DMSO). Calibration standards and quality control samples were prepared in BME medium. The concentrations of DBK-1154 and NZ-8-061 were determined with HPLC-MS/MS. Sample was prepared by mixing 25 µl of standard / quality control sample / study sample, 35 µl of internal standard (15 µM) and 440 µl of acetonitrile. Injection volume was 10 µl. After separation with a Waters SunFireTM C18 column (2.1 x 150 mm, 3.5 µm) with a gradient flow system (0.1 % formic acid in water and acetonitrile), quantitative detections were performed in multi-reaction

monitoring mode (MRM) with an AB Sciex API 4000TM triple quadrupole mass spectrometer connected to a Shimadzu Prominence HPLC system. For DBK-1154, NZ-8-061 and the internal standard, the respective precursor ions (m/z) were 533.2, 521.1 and 446.1. The fragment ions (m/z) monitored and used for quantitation were 196.1 for DBK-1154, 184.0 for NZ-8-061 and 321.1 for the internal standard. The chromatograms were processed using AB Sciex Analyst® 1.6.3 software. The fit-for-purpose validated concentration range was from 15 nM to 15 µM using quadratic regression and 1/x2 weighting. The study samples were analysed and calculated using range from 150 nM to 15 µM. Inter-assay accuracies of six quality control samples [two parallel quality control samples at three different concentration levels (450 nM, 4.5 µM and 12 µM)] ranged from 84.9 % to 112 % for DBK-1154 and from 81.9 % to 109 % for DT-061.

Caspase-3 and -7 activity

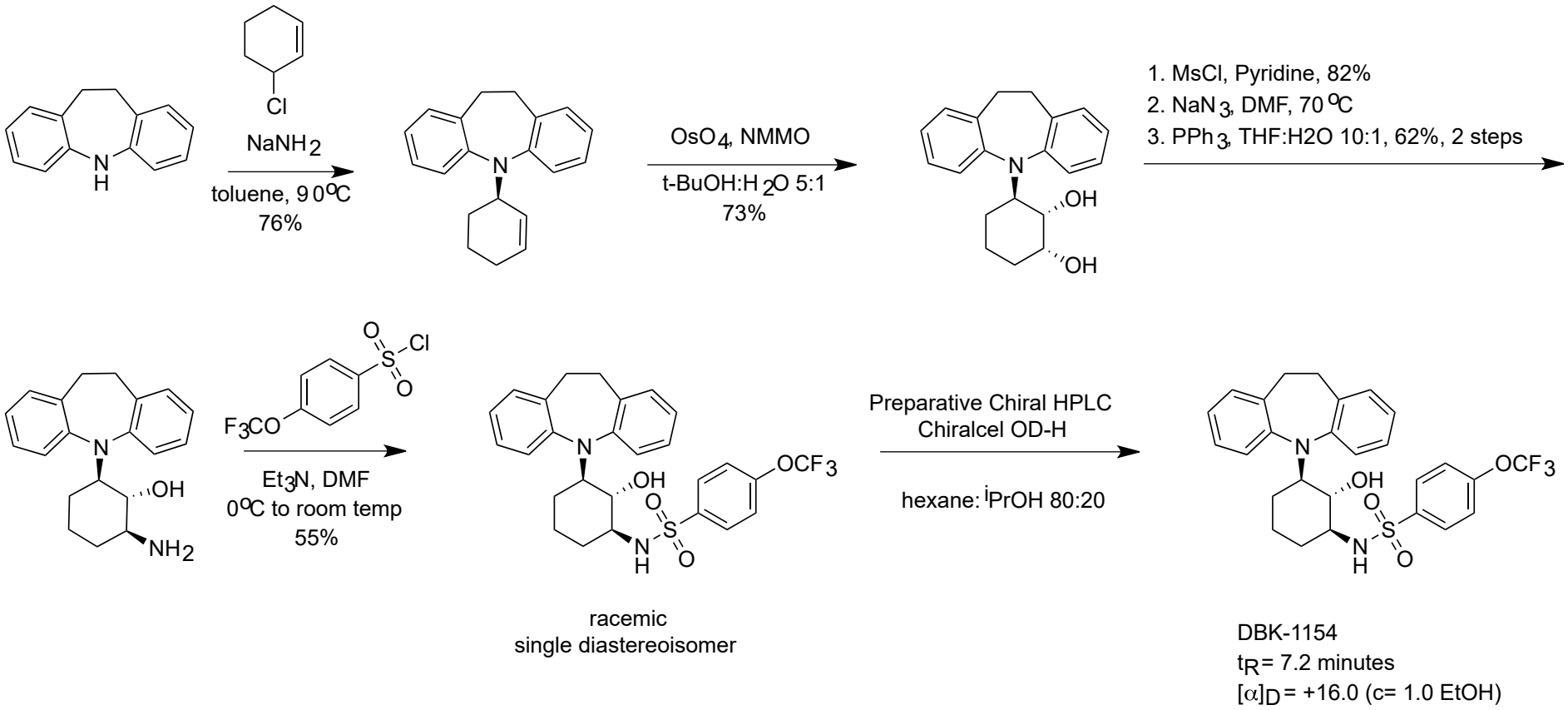
Optimized number of E98 cells (3 x 10^3 to 5 x 10^3) were seeded in white 96-well plates (Perkin Elmer) and allowed to attach. After 24 hours cells were treated with determined concentration of chemical compounds for 24-72 hours. Caspase-3 and -7 activity was measured by luminescence-based method, Caspase-Glo® 3/7 Assay (Promega), which utilize a substrate containing Caspase-3 and -7 target peptide DEVD-aminoluciferin according the manufacturer's instructions. Luminescence was read with Perkin BioTek Synergy H1. Bioluminescence was normalized and presented as a percent of the control.

Cancer cell line encyclopedia and drug sensitivity profiling

All mutations and copy number variations of U87MG, U118, A172 and T98G GB cell lines were collected from CCLE (https://portals.broadinstitute.org/ccle). For E98 data was gathered from (*27, 30*). Drug sensitivity data for U87MG, U118, A172 and T98G was gathered from

https://www.cancerrxgene.org/. Drug sensitivity of five primary patient derived glioma cell lines was gathered from the accompanying manuscript (Johansson et al., 2019).

Supplementary figure 1.



Supplementary figure 1:
Schematic presentation of the synthesis of DBK-1154. The synthesis of SMAPs is described in detail in public patent application PCT/US2015/019674.

Supplementary figure 2.

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Genetic alterations in GBM cell lines

Common genetic alterations	U87MG	A172	U118	T98G	E98
RTK					
EGFR	CV gain	Coding silent			
PDGFRA			Missense		
PDGFRB		Over expression			
MET					Mutation
PI3K					
PIK3CB		Missense			
PIK3C2A	Over expression				
PTEN	Mutation	Loss of CN	Mutation	Missense	
MAPK					
NF1	Deletion				
BRAF	Over expression				
KRAS	Under expression				
NRAS	Over expression				
P53					
TP53			Missense	Missense	
RB1					
CDKN2A	Loss of CN	Loss of CN	Loss of CN	Loss of CN	Loss of CN
CDKN2B	Loss of CN	Loss of CN	Loss of CN		
CDKN2C	Loss of CN			Loss of CN	
CCND2			Missense		
RB1		Missense			
Chromatin modifier					
ATRX	Missense				
Other					
MGMT	+++				

B

		IC50 (μM)			
		U87MG	T98G	A172	U118
EGFR	Gefinitib	**44.84**	20.32	17.63	33.00
	Osimertinib	**15.13**	12.35	4.14	5.33
VEGFR	Sorafenib	**35.93**	16.33	7.22	11.79
	Cediranib	7.27	**14.15**	1.71	8.28
MEK	Trametinib	2.72	**10.26**	1.50	2.88
MET	Foretinib	**2.65**	2.42	1.33	2.02
	Savolitinib	**13.74**	13.51	6.84	3.92
BRAF	Dabrafenib	**381.38**	161.77	52.09	341.55
mTOR	Rapamycin	0.05	0.05	0.04	**0.07**
PI3K	Alpelisib	62.61	45.46	119.21	**399.87**
	Buparlisib	3.00	**3.25**	1.23	2.51
CDK 4/6	Palbociclib	58.96	**62.88**	18.80	52.25
AKT	Uprosertib	**90.44**	23.86	15.72	26.29
	MK-2206	28.86	**60.39**	46.15	27.82

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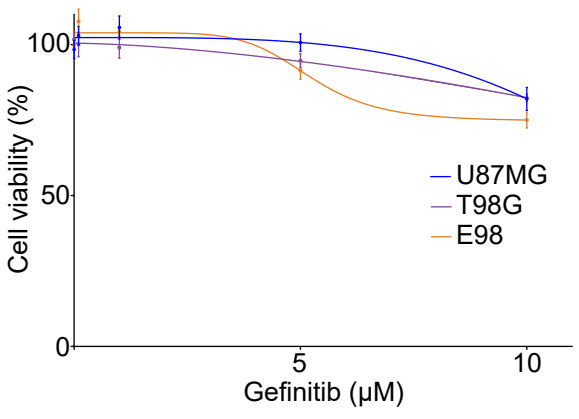

Supplementary figure 2:
A) Genomic alterations of used GB cell lines data gathered from cancer cell line encyclopaedia (https://portals.broadinstitute.org/ccle). For E98 data was gathered from (26, 30). B) IC50 values of multiple kinase inhibitors in established GB cell lines. Data was gathered from https://www.cancerrxgene.org/. The most resistant cell line showing highest IC50 for the indicated kinase inhibitor is shown in bold. C) Viability of T98G, U87MG and E98 after 72 hours treatment with 10 μM dose of EGFR inhibitor Gefitinib measured with CellTiter-Glo assay.

Supplementary figure 3.

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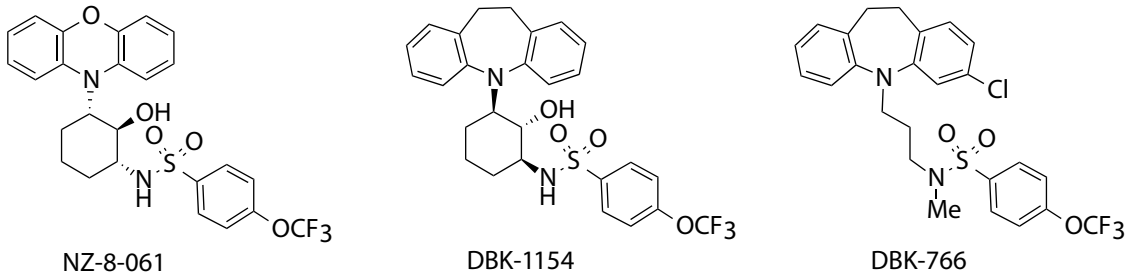

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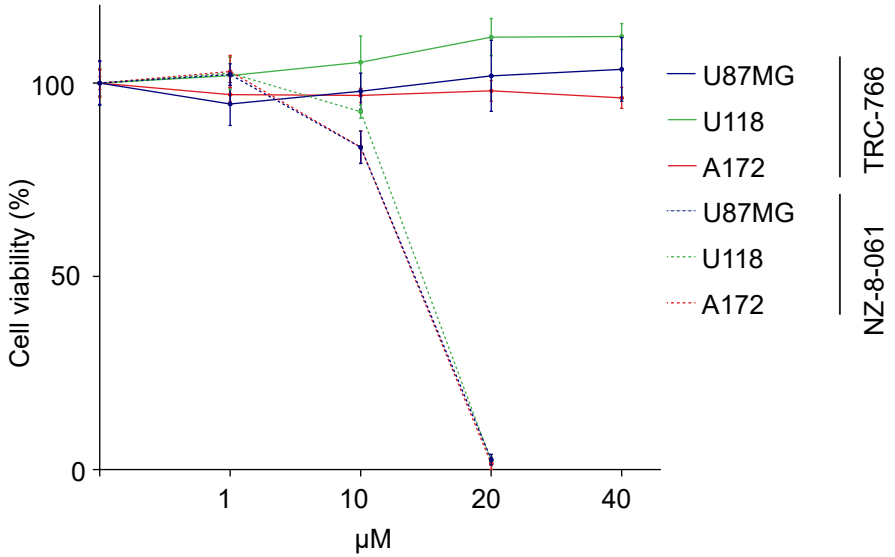

Supplementary figure 3:
A) Chemical formulas of active SMAPs NZ-8-061 and DBK-1154, and of inactive SMAP DBK-766. B) Cell viability comparison in U87MG, U1118 and A172 GBM cell lines with either NZ-8-061 or an inactive SMAP TRC-766.

Supplementary figure 4.

A

	P value of cell line for subtypes								
Subtype	U3008	U3013	U3017	U3054	U3173	U3180	U3137	U3213	U3179
'Proneural_pval'	1	0.003	0.996	0.052	0.101	0.999	0.728	0.698	0.718
'Classical_pval'	0.106	0.996	0.006	1	0.946	0.001	0.258	0.543	0.462
'Mesenchymal_pval'	0.783	0.970	0.952	0.097	0.023	0.996	0.029	0.002	0.996

B

Subtypes of primary glioma cell lines	
U3008	Classical
U3013	Proneural
U3017	Classical
U3054	Proneural
U3140	Proneural
U3173	Mesenchymal
U3179	Classical
U3180	Classical
U3213	Mesenchymal

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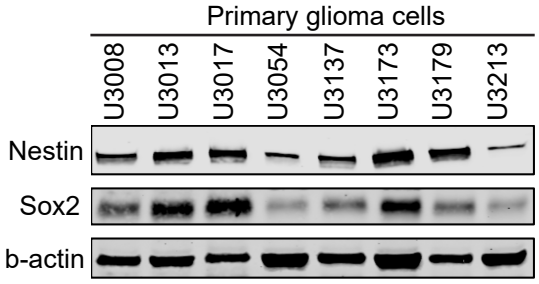


Supplementary figure 4:
A) P values represent gene expression profile similarity of the indicated patient derived primary glioma cells to the established molecular signature for each GB subtype according to (33). The lowest p-value used for subtype calling in figure 2G is shown in red. B) Subtype classification of patient-derived glioma stem cell lines based on copy number alterations in A). C) Expression levels of stem cell markers (Sox2 and Nestin) in patient-derived primary glioma cell lines. Please see Supplementary material of full uncut Western blots.

Supplementary figure 5.

A

Copy number alterations in patient-derived glioma stem cell lines

	U3008	U3013	U3017	U3054	U3140	U3173	U3179	U3180	U3213
'ATRX'	0.042	0.306	-0.534	0.082	0.063	-0.13	-0.565	-0.634	0.021
'BRAF'	0.386	0.356	0.405	0.413	0.358	0.376	0.415	0.398	0.408
'CCND2'	0.047	0.244	0.065	0.077	0.076	0.009	0.062	0.039	0.039
'CDKN2A'	-1.996	0.093	-1.3	-0.309	-1.835	-0.03	-1.853	-1.97	-2.162
'CDKN2B'	-1.996	0.093	-1.3	-0.309	-1.835	-0.03	-1.853	-1.97	-2.162
'CDKN2C'	0.041	-0.142	-0.457	0.024	-0.132	0.043	0.052	0.033	-0.106
'EGFR'	0.38	0.345	0.396	0.522	0.3	0.34	0.638	0.434	0.407
'KRAS'	0.068	0.275	0.065	0.13	0.058	-0.009	0.038	0.009	0.039
'MET'	0.403	0.356	0.392	0.61	0.437	0.299	0.294	0.32	0.408
'MGMT'	-0.521	-0.146	-0.482	-0.328	-0.535	-0.279	-0.521	0.036	-0.434
'NF1'	0.046	0.236	0.075	0.204	0.052	-0.065	-1.97	0.073	0.037
'NRAS'	0.061	0.052	0.066	0.026	-0.185	0.186	0.046	-0.001	-0.106
'PDGFRB'	0.06	0.043	0.05	-0.095	0.049	-0.026	0.043	0.019	0.022
'PIK3C2A'	0.041	0	0.067	0.017	0.008	-0.064	0.05	0.013	0.046
'PIK3CB'	0.03	0.109	0.057	-0.075	0.072	0.139	0.046	-0.015	-0.082
'PTEN'	-0.326	0.001	-0.482	-1.735	-1.73	-1.737	-0.369	-0.407	-0.434
'RB1'	0.033	-0.39	-0.465	-0.052	-0.167	-0.411	0.035	0.016	0.035
'TP53'	0.041	0.236	0.043	-0.378	0.034	-0.026	0.046	0.61	0.002

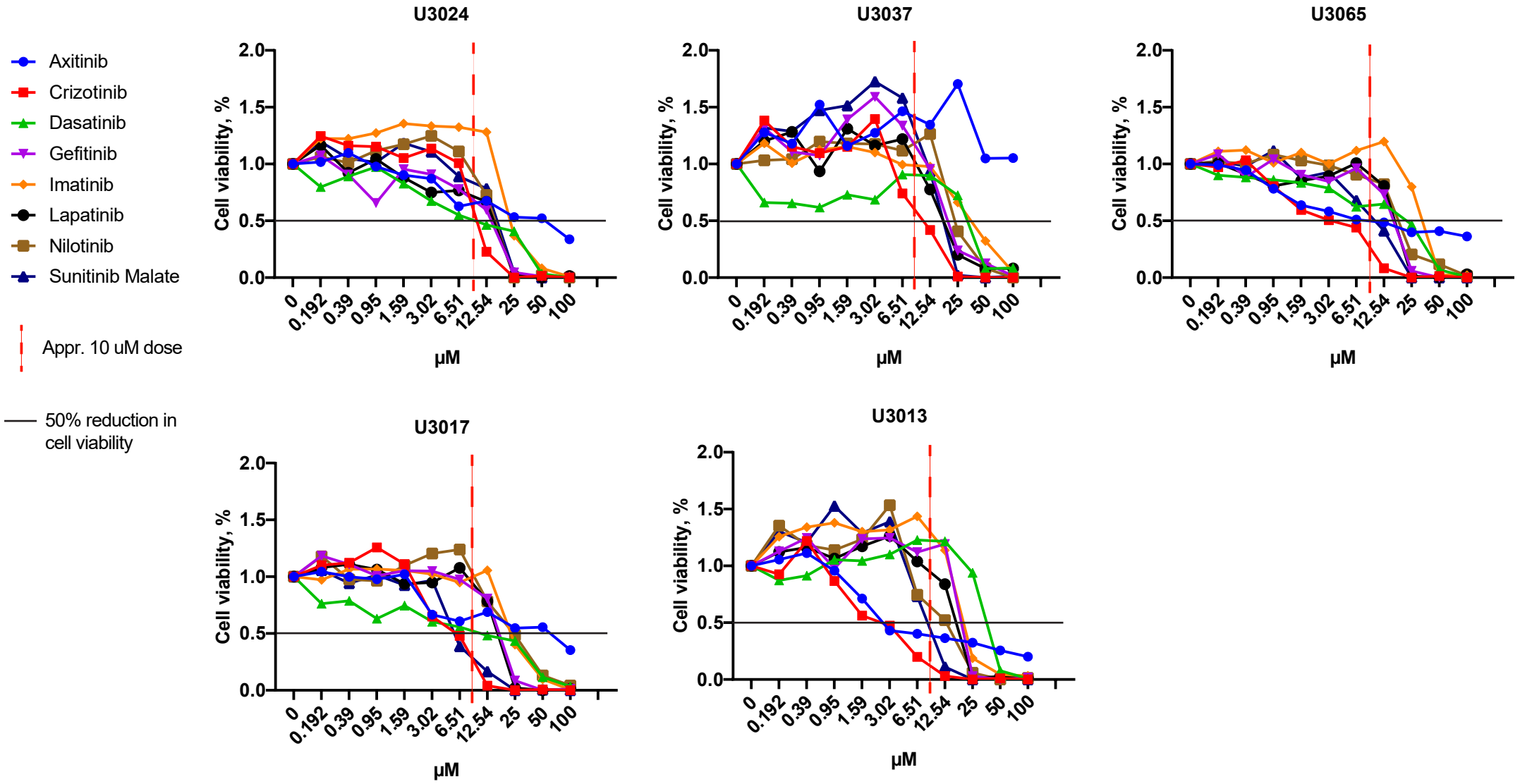
B

Point mutations in patient-derived glioma stem cell lines

Gene name	U3008	U3013	U3017	U3054	U3140	U3173	U3179
'ATRX'	0	0	0	0	0	0	0
'BRAF'	0	0	0	0	0	0	0
'CCND2'	0	0	0	0	0	0	0
'CDKN2A'	0	0	0	0	0	0	0
CDKN2B'	0	0	0	0	0	0	0
'CDKN2C'	0	0	0	0	0	0	0
'EGFR'	0	0	0	0	0	0	0
'KRAS'	0	0	0	0	0	0	0
'MET'	0	0	0	0	0	0	0
'MGMT'	0	0	0	0	0	0	0
'NF1'	0	0	0	0	0	0	0
'NRAS'	0	0	0	0	0	0	0
'PDGFRB'	0	0	0	0	0	0	0
'PIK3C2A'	0	0	0	0	0	0	0
PIK3CB'	0	0	0	0	0	0	0
'PTEN'	0	0	0	0	0	0	0
'RB1'	0	0	0	0	0	1	0
TP53'	0	1	0	1	0	1	0

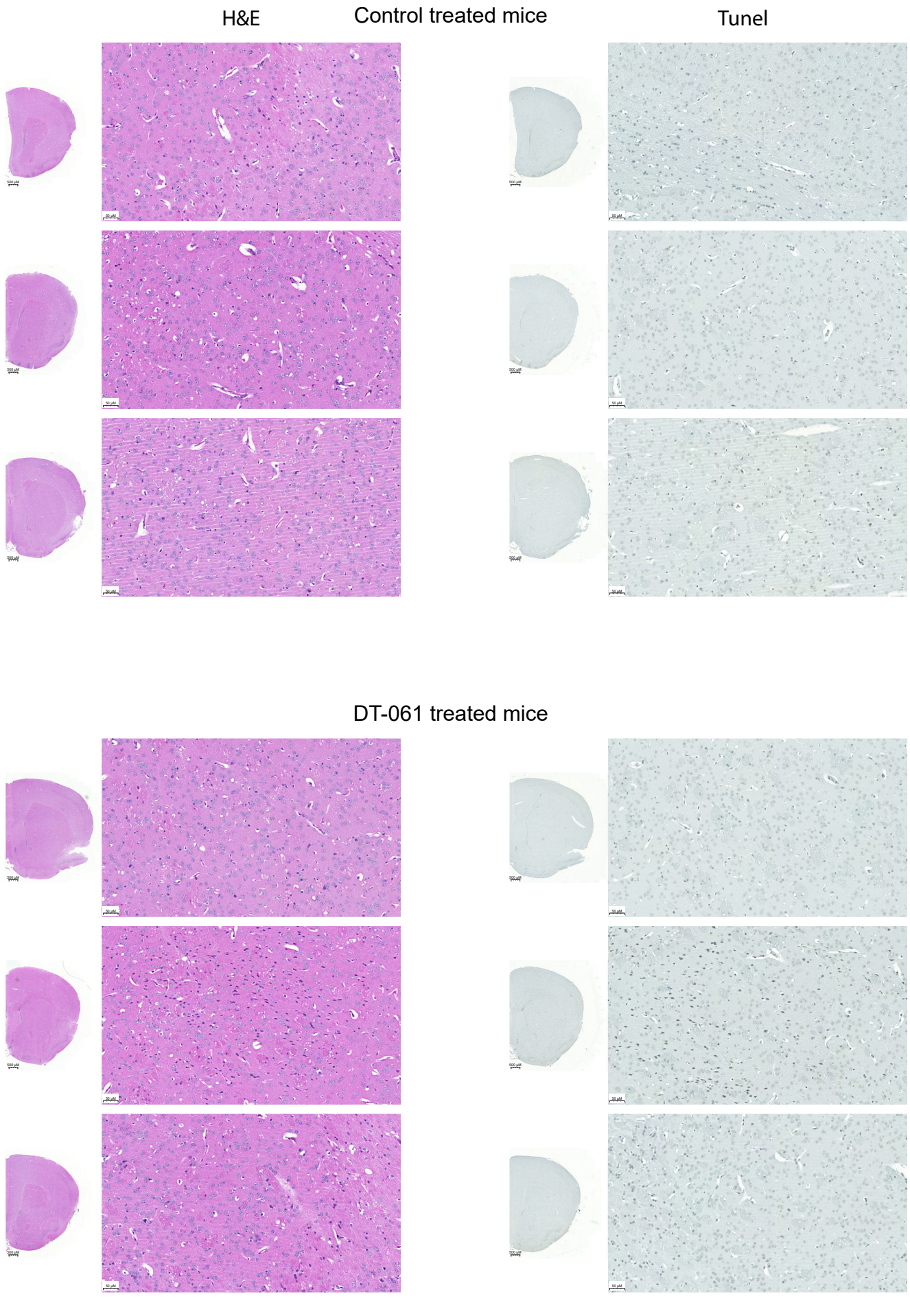
Supplementary figure 5:
A) Copy number alterations in patient-derived primary glioma cells. Numbers are log DNA content from each gene region, as determined from segmented data from Affymetrix Cytscan HD arrays. Gene regions are defined as gene start, end coordinates in the hg19 annotation. Numbers are log2 average signals of the chromosomal segment spanning that gene locus. -1 bi-allelic loss, -0.5 monoallelic loss, 0 normal copy number, +0.5 or greater gain. Affymetrix Cytoscan HD array method. (data from accompanying manuscript Johansson et al., 2019). B) Point mutations in patient-derived GB stem cell lines: 0 = no detected somatic nonsynonymous mutation 1 = at least one somatic nonsynonymous mutation in that gene locus. Ion Torrent whole exome sequencing, consensus calling with 4 callers, Annovar (data from accompanying manuscript Johansson et al., 2019).

Supplementary figure 6.



Supplementary figure 6:
Dose-dependent cell viability inhibition after treatment of patient-derived primary glioma cells cultured in serum-free neural stem cell medium with indicated kinase inhibitors.

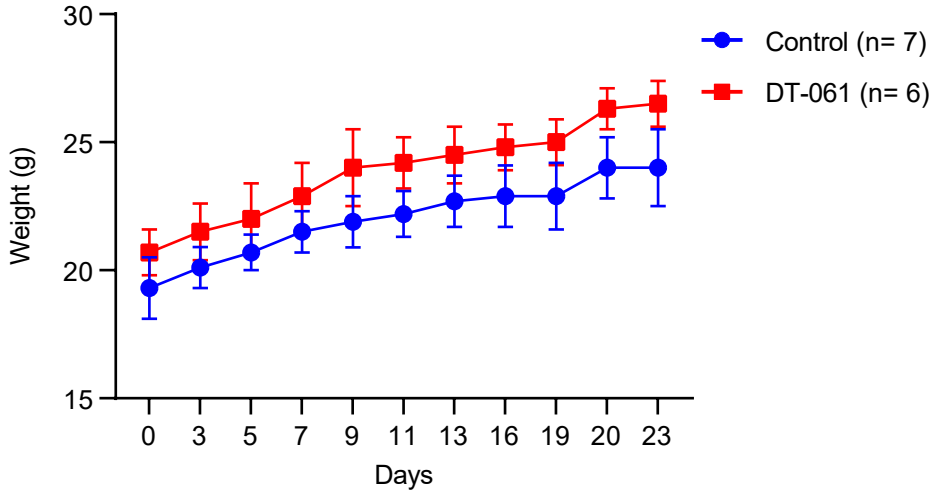
Supplementary figure 7.



Supplementary figure 7:
H&E and TUNEL staining's of mice brains after treatment with either NZ-8-061 (50 mg/kg twice a day) or vehicle for 21 days. BALB/cOlaHsd-Foxn1nu mice were used.

Supplementary figure 8.

A



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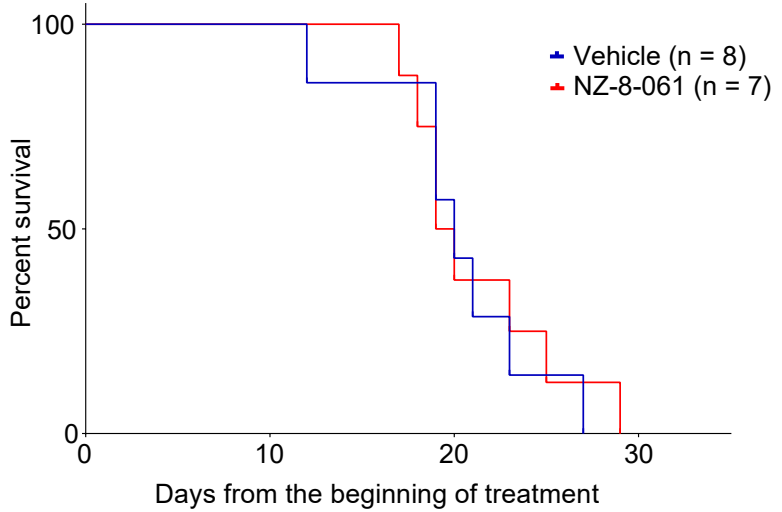

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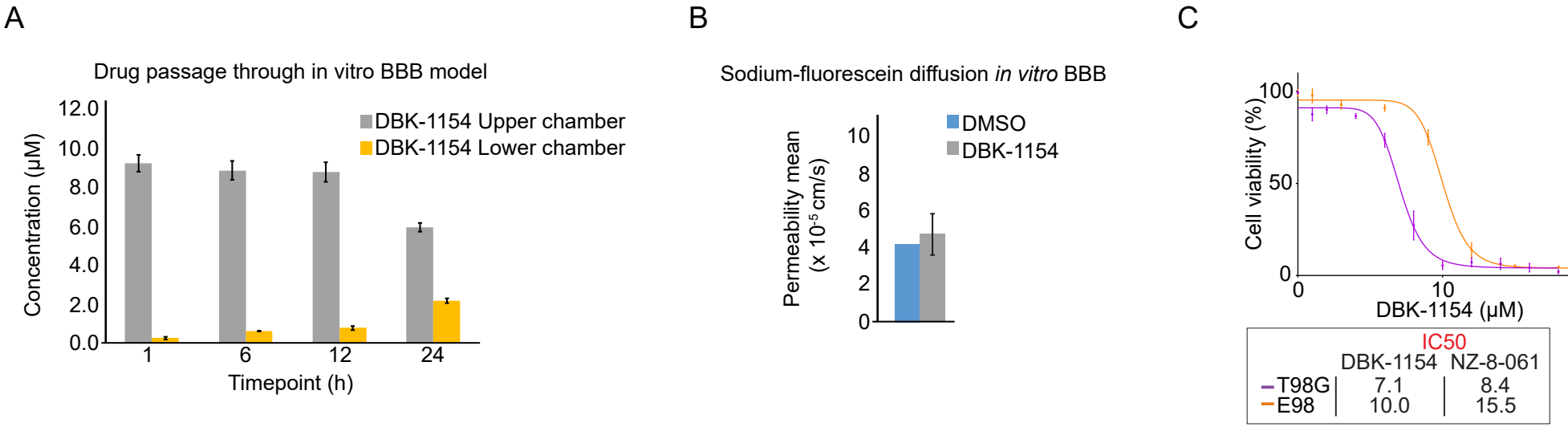
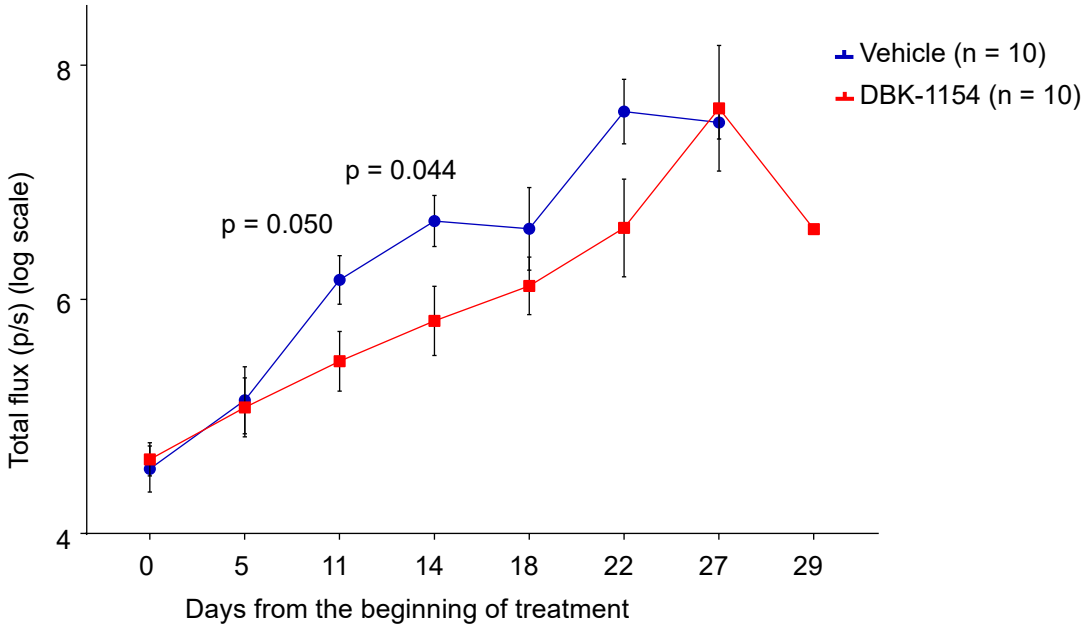
Compound ID	Route	Dose	T½ hr	Tmax hr	Cmax ng/mL	AUC hr.ng/mL	CL mL/hr/kg	%F	Blood-brain ratio
DBK-1154	i.v.	1 mg/kg	2.1	-	1478	495	1950		
DBK-1154	p.o. (soln.)	100 mg/kg	5.1	2.0	2253	11702	-	23	1:1
DBK-1154	p.o. (susp.)	100 mg/kg	3.6	4.0	1200	7130	-	14	1:1

For Mr = 500 g/mol, 1000 ng/mL = 2.0 micromolar
Tmax; time to Cmax, Cmax; maximum serum concentration, AUC; Area under curve, CL; clearance

Supplementary figure 8:
A) Overall survival of mice after vehicle or 30 mg/kg NZ-8-061 treatment from figure 3. B) Mouse in vivo pharmacokinetic parameters (T½ hr, Tmax hr, Cmax ng/mL, AUC hr.ng/mL, CL mL/hr/kg and %F) after 1 mg/kg or 100 mg/kg dosage via p.o. (solution or suspension) or i.v. of DBK-1154.

Supplementary figure 9.





Supplementary figure 9:
A) Timepoint (1, 6, 12 and 24 hours) concentration measurements of DBK-1154 passage through the in vitro BBB after 15 µM dosage on the upper chamber. Data shown are means from two replicates ± SD. B) Sodium-fluorescein diffusion through the in vitro BBB after 24 hours pretreatment with 15 µM NZ-8-061 on the upper chamber. Fluorescence signal from sodium-fluorescein was measured after 15 minutes. Data shown are means from two replicates ± SD. C) Cell viability after DBK-1154 treatment in GB cell lines with indicated concentrations. D) Bioluminescence follow up during the intracranial E98 in vivo model during vehicle or 100 mg/kg DBK-1154 treatment. Data shown are means from 10 replicates ± SD, *, < 0.05 by Student t test. Xenograft tumors were established in nude mice after intracranial injection of E98 GMB cells. When tumors were visible with bioluminescence, mice were randomized (2 groups of 10) based on bioluminescence signal before starting the treatment.

Full uncut western blots

Figure 2A, B

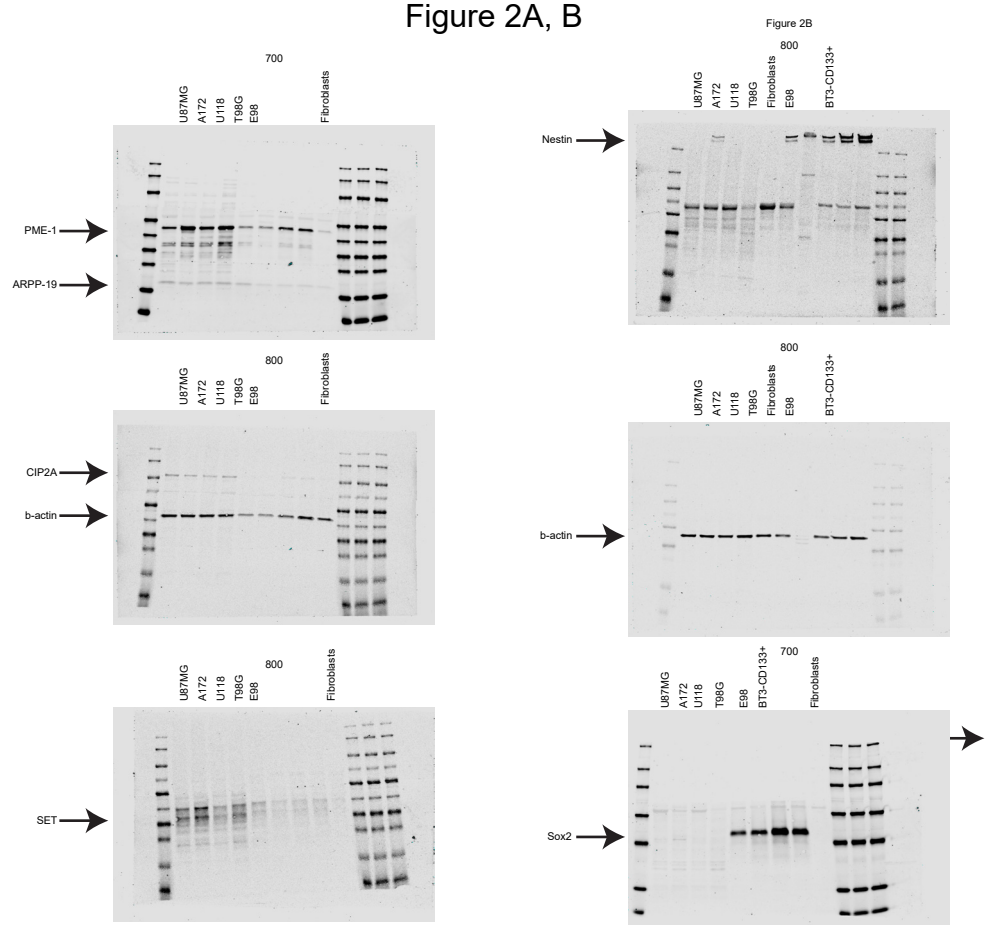


Figure 2F & Supplementary Figure 4C